Research in Biotechnology, 3(3): 26-36, 2012 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

In silico analysis of lipopolysaccharide and β-1, 3-glucan binding protein (LGBP) gene from the haemocytes of Indian white shrimp *Fenneropenaeus indicus*

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Lipopolysaccharide and β-1,3-glucan binding protein (LGBP) gene are involved in the pattern recognition mechanism of invertebrates, it induces the cell and humoral mediated immune responses like encapsulation, phagocytosis, nodule formation, clotting, synthesis of antimicrobial peptides and activation of the prophenoloxidase (proPO) system. The current study focuses to model the three-dimensional structure of novel immune related gene LGBP from the Indian white shrimp *Fenneropeneaus indicus* (*F.indicus*) by *in silico* homology modeling and its motif prediction. *Fenneropeneaus indicus* lipopolysaccharide and β-1,3-glucan binding protein (*Fein*-LGBP) consists of glycosylated regions which come under the glucanase family. Two conserved putative integrin-binding motif (cell adhesion sites), bacterial glucanase motif (GM) and two polysaccharide recognition motifs for the polysaccharide binding motif (PsBM) and β-glucan recognition motif (β-GRM) were conserved in the novel sequences of *Fein*-LGBP. Prediction of motifs, patterns, disulfide bridges and secondary structure were performed for functional characterization of the *Fein*-LGBP. Three dimensional structure of the *Fein*-LGBP was generated by Modeller9V8, Swiss Model and validated using NIH server. Results revealed that the modelled structure of *Fein*-LGBP was 75.7% of residues in allowed region. Theoretical model of *Fein*- LGBP facilitates to the discovery of new synthetic immune related peptides, agonists that could be useful to understand the mechanism of LGBP involvement in the prophenoloxidase activating system of crustaceans. The tertiary structure prediction of the immune related gene *Fein*- LGBP will assist to explore more knowledge in immune system of crustaceans.

Keywords: LGBP, *F. indicus*, crustaceans, Comparative modeling, Motif, Ramachandran plot

Invertebrate immune system must rely on non-self-recognition molecules to ensure efficient defense responses against infectious pathogens that continuously threaten their survival. Innate immune reactions are the first line of defense for both vertebrates as well as invertebrates and are initiated by several kinds of non-self pathogen-associated molecular patterns (PAMP) particularly lipopolysaccharide β

Received: 5.4.2012; Accepted: 18.6.2012

1, 3-glucan binding protein (LGBP). LGBP plays the vital role in defense mechanism of invertebrates immune system. Structural studies of LGBP proteins showed the active site was occupied with (Arg–Gly–Asp) amino acids, which are responsible for the pattern recognition mechanism throughout adverse conditions. Basically LGBP is a glycosylated protein, which has the ability to bind with the glycosylated substrates like carbohydrate moieties. LGBP consists of pattern recognition domains which are responsible for the proPO activation. ProPO was activated by the enzyme serine proteinase followed by LGBP. Guanine nucleotide binding proteins (GNBP) otherwise known as LGBP, was originally purified from the silkworm *Bombyx mori* (Lee *et al.,*1996) *Drosophila melanogaster* (Kim *et al.,* 2000). It was also purified from mealworm and found to be play vital role in proPO activation. In annelid *Eisenia fetida*, coelomic cytolytic factor-1 (CCF-1) has GNRP protein that binds with LPS and β-glucans (Beschin *et al.,* 1998; Bilej *et al.,* 2001; Lee et al., 2000). Moreover, some of the insect GNBPs are not β-GBP but PGRPs (Peptido Glycan Recognition Proteins) (Dziarski *et al.,* 2006; Wang *et al.,* 2006). Characterization of β-1, 3-glucan binding protein from *Manduca sexta,* and aggregate with bacteria, fungi and to stimulate proPO activation (Yu *et al.,* 2003; Zhang *et al.,* 2003). In mammalian cells toll like receptor -4 (TLR-4) recoginizes the lipopolysaccraides and it stimulate the activation of downstream signalling cascade (Mdezhitov *et al.,* 1997; Miyake, 2003). In freshwater crayfish *Pacifastacus leniusculus* LGBP has involved in the activation of the proPO cascade was divulged (Lee *et al.,* 2000). βG–βGBP complex induces degranulation of hemocytes, consequently enhances activation of the prophenoloxidase (proPO) system which brings about synthesis of melanin through the oxidation of phenols (Soderhall *et al.,*1998; Duvic *et al.,*1992; Vargas-Albores *et al.,*2000)

β-GBP was identified and characterized from tiger shrimp *Penaeus monodon* (Sritunyalucksana *et al.,* 2002), kuruma shrimp *Marsupenaeus japonicus* (Lin *et al.,* 2008), white shrimp *L. vannamei* (Romeo-Figueroa *et al.,*2004), and lobster *Homarus gammarus* (AJ583519), blue shrimp *Litopenaeus stylirostris* (Roux *et al.,* 2002), white shrimp *L. vannamei* (Cheng *et al.,*2005), *P. leniusculus* (Liu *et al.,*2008) and Chinese shrimp *F. chinensis* (Xin-Jun Du *et al.,*2006). However, *in silico* analysis of the LGBP gene and the character prediction are scanty. Hence, in the present study, we constructed the model structure for *Fein*-LGBP using known structural templates and describe its structural features to understand molecular function.

Materials and Methods

Indian white shrimp *Fenneropenaeus indicus* ranged from 15.7 to 23.2 g, were obtained from the coastal area of Nagapattinam to Chennai, Tamil Nadu, India. *F.indicus* were stocked and maintained in FRP tanks with flow-through sea water (35 % salinity) at 28°C and fed twice daily with a formulated shrimp diet. The haemolymph was collected from the ventral sinus of an individual shrimp using anti coagulant solution (0.45 M NaCl, 0.1M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 7.5, Osmolality 780 mOsm kg^{-1}) and immediately centrifuged at 500 g at 4˚C for 20 min to separate the hemocytes from the plasma. The resulting haemocyte pellet was used for total RNA isolation. Molecular approaches were used to clone the propheloxidase stimulating LGBP gene in the hemocytes of Indian white shrimp *F. indicus*. The full length sequence of *Fein*-LGBP gene was determined by Reverse Transcriptase PCR, cloning and sequencing of overlapping PCR and rapid amplification of cDNA ends (RACE) method. The same sequence was used for the further studies *in silico* homology modeling analysis studies.

Theoretical calculations of physico-chemical characterization

The physico-chemical characterizations of the parameters were computed by ProtParam (Xin-Jun Du *et al.,* 2006). It include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient (Gasteiger, 2005), estimated half-life, instability index (Gill *et al.*,1989), aliphatic index (Guruprasad *et al.*,1990) and grand average of hydropathicity (GRAVY) (Ikai,1980).

Functional characterization-DAS

Potential transmembrane segments and hydrophobicity values were predicted by DAS and the TMHMM server.

Secondary structure prediction

SOPMA (Geourjon *et al.,* 1995) and GOR were employed for calculating the secondary structural features of the pattern recognition protein *Fein*-LGBP.

Comparative modeling

Modeller 9V8: Modeller was used for homology or comparative modeling of protein three-dimensional structures. Alignment of a sequence to be modeled is provided with known related structures and modeller automatically calculates a model containing all non-hydrogen atoms. Modeller (Sali *et al.,* 1993) implements comparative protein structure modeling by satisfaction of spatial restraints, and can perform many additional tasks, including de novo modeling of loops in protein structures, optimization of various models of protein structure with respect to a flexibly defined objective function, multiple alignment of protein sequences and/or structures, clustering, searching of sequence databases, comparison of protein structures, etc. The sequence of *Fein*-LGBP was searched against selection of the related homologues of query sequence in PDB. The homology modelling requires sequences of known 3D structure and the target having above 35% of similarity. In order to confirm the selection pair wise alignment of template and target was performed.

Selection of the template

Modeller is a computer program used for comparative protein modelling (Fiser *et al.,* 2000). Comparative modelling or Knowledge based modelling consist of four steps – fold assignment i.e based on the identity sequences were chosen in the FASTA with for the PDB databank on NCBI. The chosen templates were the sequence from the latest version of the PDB databank with the lowest expected value and highest score after four iterations, building a model based on the alignment with the template and predicting the accuracy of the model (Eswar *et al.,* 2005).

Alignment

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as identify members of gene families. To ensure the high accuracy of the structure, the target and the template sequence can be aligned.

Model building and refinement

Although the theory behind building a protein homology model is complicated, using available programs is relatively easy. Several modelling programs are available, using different methods to construct the 3D structures. In segment matching methods, the target is divided into short segments, and alignment is done over segments rather than over the entire protein. Satisfying spatial restraints is the most common method. It uses either distances or optimization techniques to satisfy the spatial restraints. The method is implemented using the popular program, Modeller and which includes the CHARMM energy terms that ensure valid

stereochemistry is combined with spatial restraints (Marti-Renom *et al.,* 2000; Brooks *et al.,* 1993). The academic version of MODELLER9V8 (Sali *et al.*, 1993) was used for 3D structure generation based on the information obtained from sequence alignment.

Validation

The best validation combines common sense, biological knowledge and results from analytical tools. Most refinement involves adjusting the alignment. We used PROCHECK (Laskowski *et al.,* 1993) to calculate the main-chain torsion angles, i.e. the Ramachandran plot (Ramachandran *et al.,* 1963), for our predicted structures. Among the three models were predicted using different templates among those the one that shows the good resolution factor and R-factor was used as a template and evaluated by Procheck (http: //www.biotech. ebi.ac.uk/) performing full geometric analysis with a resolution of 1.5 Å. The validation for structure models obtained from the three software tools was performed by using PROCHECK (Laskowski *et al.*1996). The models were further checked with WHAT IF (Vriend, 1990). Structural analysis was performed and figures representations were generated with Swiss PDB Viewer (Guex *et al.,* 1997). Ramachandran plot statistics was used to evaluate the best model. The root mean square deviation (RMSd) values were calculated using the modeller by fitting the carbon backbone of the predicted. Finally the all-atom models were subjected to a short run of energy minimization by using AMBER (Baker et *al.*, 2001) to relieve unfavorable steric interactions and to optimize the stereochemistry. Fold recognition energy minimization was done by using several tools available in SPDBV. Some of them are energy minimization; loop building, Side chain fixing setting phi/psi angles, fixing the clashing amino acids.

Result and Discussion

Physico chemical characterizations of *Fein* LGBP were computed using Expasy's ProtParam tool was recorded (Table 1). The calculated isoelectric point (pI) of *Fein*- LGBP was 5.8, in this pH the proteins are stable and compact. The computed pI value of *Fein*-LGBP was less than 7 (pI< 7) indicates that these proteins were considered as acidic. The computed isoelectric point (pI) will be useful for developing buffer system for purification by isoelectric focusing method.

Table1: *Fein*-LGBP Physicochemical characterization by Protparam

Protein name	Accession Number	Sequence length	M.wt	PI	-R	+R	EC	II	AI	GRAVY
Fein-LGBP	HQ318720	1372	45872.8	4.99	54	37	29370	39.73	60.66	-.595

Parameters computed using Expasy's ProtParam tool. Theoretical isoelectric point (pI), molecular weight (M.wt), total number of positive (+R) and negative residues (-R), Extinction coefficient (EC), Instability index (II), aliphatic index (AI) and Grand average hydropathy (GRAVY) of *Fein*-LGBP was depicted in table 1.

The extinction coefficient indicates how much light a protein absorbs at a certain wavelength. It is useful to have an estimation of this coefficient for following a protein which a spectrophotometer when purifying it and possible to estimate the molar extinction coefficient of a protein from knowledge of its amino acid composition. From the molar extinction coefficient of tyrosine, tryptophan and cystine (cysteine does not absorb appreciably at wavelengths >260 nm, while cystine does) at a given wavelength. Although Expasy's ProtParam computes the extinction coefficient for 278, 279, 280 and 282 nm wavelengths, 280 nm is

favoured due to the proteins absorb strongly. Extinction coefficient of *Fein*-LGBP homologue at 280 nm is ranging from 45840 from 46715 M^{-1} cm^{-1} with respect to the concentration of Asn, Asp and Ala. The high extinction coefficient of *Fein*-LGBP indicates that presence of catalytic triad of Arg167, Gly127 and Asp178. The computed extinction coefficients help in the quantitative study of protein–protein and protein–ligand interactions in solution.

The instability index provides an estimate of the stability of our protein in a test tube. Statistical analysis of unstable and stable proteins difference was revealed by instability index. Aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (ala, val, iso, and leu). It may be regarded as a positive factor for the increase of thermo stability of globular proteins. There are certain dipeptides, the occurrence of which is significantly different in the unstable proteins compared with those in the stable ones. This method assigns a weight value of instability. Using these weight values it is possible to compute an instability index (II). A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable. The instability index value for the immune related proteins was found to be ranging from 39.73.

The aliphatic index (AI) which is defined as the relative volume of a protein occupied by aliphatic side chains (A, V, I and L) is regarded as a positive factor for the increase of thermal stability of globular proteins. The very high aliphatic index of all LGBP sequences indicates that these proteins may be stable for a wide temperature range. The lower thermal stability of *Fein*-LGBP was indicative of a more flexible structure when compared to other protein. The Grand Average hydropathy (GRAVY) value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. The GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. GRAVY indices of *Fein*-LGBP are ranging from - 0.595. This low range of value indicates the possibility of better interaction with water. As disulphide bridges play an important role in determining the thermo stability of these proteins. CYS_REC was used to determine the Cysteine residues and disulphide bonds (Fariselli *et al.,* 2001). Cystein residues are exceptionally present in the *Fein*-LGBP gene (Ferre *et al.,* 2005).

Table 2: Transmembrane regions identified by TMHMM server. This amino acid sequence is of a MEMBRANE PROTEIN which has 1 transmembrane helix.

Name of the Protein	Accession Number	Sequence Length	Expected Number of AAs in TMHs	Expected Number of first 60 AAs in TMHs	Total prob of 'N'
Fein-LGBP	HQ318720	407	0.36478	0.1892	0.04695

Functional characterization of LGBP

Functional analysis of the *Fein*-LGBP includes prediction of transmembrane region, disulfide bond and identification of important motifs. DAS distinguishes between membrane and soluble proteins from amino acid sequences, and predicts the transmembrane helices for the former. The transmembrane regions and their length were tabulated in Table 2. The server Expasy (Brooks *et al.,* 1993) classifies *Fein*-LGBP as membrane protein and other

proteins as soluble proteins. DAS server has identified one transmembrane region in these proteins. The transmembrane regions are rich in hydrophobic amino acids. Table 3 showed the output of Prosite that was recorded in terms of the length of amino residues of protein with specific profiles and patterns. Secondary structure analysis revealed alpha helixes were dominated among secondary structure elements followed by random coils, extended strand and beta turns for all sequences (Table 4).

Table 3: Functional characterization of proteins of *Fein*-LGBP at Prosite.

Name of the Protein	Accession No	Motif found	Position of the Protein	Description
Fein-LGBP	HQ318720	No hits	--	Trans membrane Protein

Table 4: Secondary structure prediction of *Fein*-LGBP by SOPMA & GOR

Secondary structure	SOPMA	GOR
Alpha helix	8.60%	15.48%
310 helix	0.00%	0.00%
Pi helix	0.00%	0.00%
Beta bridge	0.00%	0.00%
Extended strand	24.32%	26.29%
Beta turn	7.37%	0.00%
Bend region	0.00%	0.00%
Random coil	59.71%	58.23%
Ambiguous states	0.00%	0.00%
Other states	0.00%	0.00%

Comparative modeling of *Fein*-LGBP & validation of the model

According to the identity three templates were chosen. Based on the Ramachandron Plot value and, over all quality factor best one model was selected as suitable template. The stereo chemical quality of the predicted models and accuracy of the protein model was evaluated after the refinement process using Ramachandran Plot Map calculations computed with the PROCHECK program. The assessment of the predicted models generated by modeler was shown in Fig 1. The main chain parameters plotted are Ramachandran Plot quality, peptide bond planarity, absence of ionic bond interaction, main chain hydrogen bond energy, C-alpha chirality and over-all R factor. In the Ramachandran Plot analysis, the residues were classified according to its regions in the quadrangle. Stereo chemical structure was observed through Pymol (Fig 2). The red regions in the graph indicate the most allowed regions whereas the yellow regions represent allowed regions. Glycine is represented by triangles and other residues are represented by squares. The result revealed that the modelled structure for *Fein*-LGBP was 75.7% of residues in allowed region. The distribution of the main chain bond lengths and bond angles were found to be within the limits for these proteins. Such figures assigned by Ramachandran plot represent a good quality of the predicted models. The modelled structures of *Fein*-LGBP protein was also validated by other structure verification servers WHAT IF. Standard bond angles of the model were determined by using WHAT IF and the results were shown in Table 5. The analysis revealed RMS Z-scores were almost equal to 1 suggesting high model quality. The predicted structures conformed well to the stereochemistry indicating reasonably good quality.

Table 5: Ramachandran Plot calculation and comparative analysis of the models from Modeller computed with the PROCHECK program

Sl. no	Plot statistics	values
1	Residues in most favoured regions [A,B,L]	75.7%
2	Residues in additional allowed regions [a,b,l,p]	18.2%
3	Number of non-glycine and non-proline residues	100.0%
4	Number of end-residues (excl. Gly and Pro)	2
5	Number of glycine residues (shown as triangles)	31
6	Number of proline residues	19

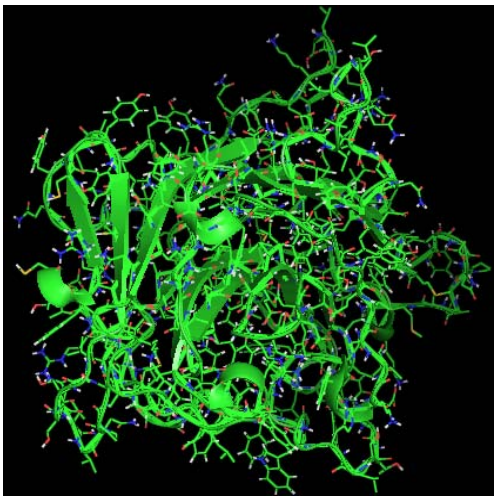

Fig 1: *Fein*-LGBP gene was modeled using the software MODELLER9V8, visualization was done by using Pymol.

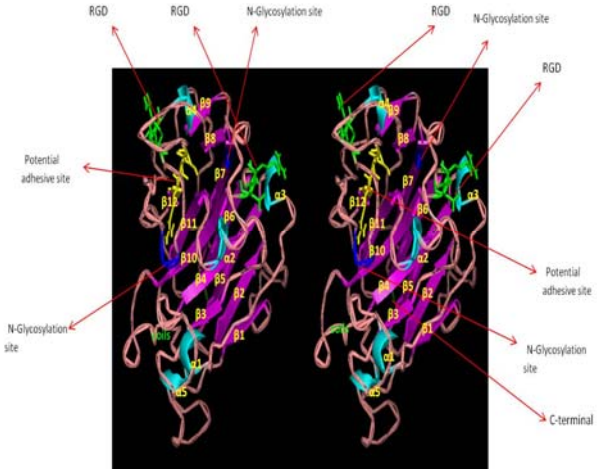

Fig 2: Stereo chemical view of *Fein*- LGBP.

To validate the homology modeled *Fein*-LGBP structure, a Ramachandran plot was drawn and the structure was analyzed by PROCHECK, a well known protein structure checking program. It was found that the phi/psi angles of 90% of residues fell in the most favored regions, 6.8% residues fell in the additional allowed regions, and 3.2% fell in generously allowed regions; none of the residues fell in the disallowed conformations (Fig 3). WHAT IF and PROCHECK were used to confirm the results with NIH server. These observations indicate that an increase in the number of bad dihedral angles of the modeled structure had occurred. This may be due to MD (Molecular Dynamics) simulation causing an unfavorable dihedral angle, allowing the protein to overcome high energy barriers.

Analysis of Model

The Discrete Optimized Protein Energy (DOPE) is a statistical potential used to assess homology models in protein structure prediction. DOPE is based on an improved reference state that corresponds to non-interacting atoms in a homogeneous sphere with the radius dependent on a sample native structure; it thus accounts for the finite and spherical shape of the native structures) method is generally used to assess the quality of a structure model as a whole. It is implemented in the popular homology modeling program MODELLER

and used to assess the energy of the protein model generated through many iterations by MODELLER, which produces homology models by the satisfaction of spatial restraints, DOPE is implemented in Python and is run within the MODELLER environment (Sali *et al.*, 1993; Eramian *et al.*, 2006; John *et al.*, 2003; Melo *et al.*, 2002; Marti-Renom *et al.*, 2000).

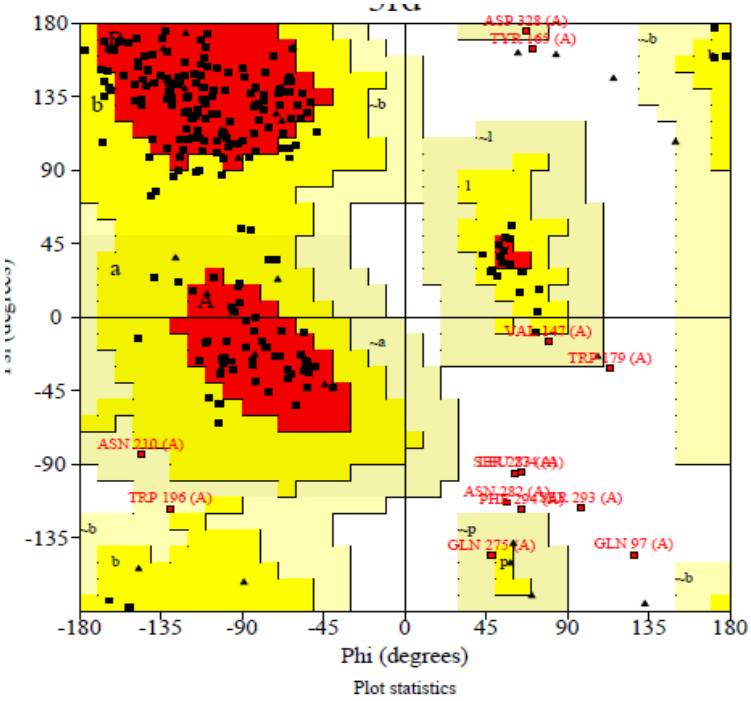

Fig 3: Ramachandran plot validation for the immune related protein *Fein*-LGBP

Motif prediction

Domain prediction of *Fein*-LGBP consist of glycosylated regions it also comes under the region between 163-296 glucanase family along with it has the number of catalytic triads responsible for Peptidoglycan Recognition Protein (PGRP) in crustaceans. Two conserved putative integrin-binding motif bacterial glucanase motif (GM) and two polysaccharide recognition motifs for the polysaccharide binding motif (PsBM) and β-glucan recognition motif (β-GRM) in the region between 151-400 were observed in the novel sequences of *Fein*-LGBP. This Integrin Binding Motifs are specific feature for the crustaceans LGBP to recognize the pathogen, where three residues Arg167, Gly127 and Asp178 form an electronegative area, it has the glucanase activity due to its bacterial glucanase site presence, moreover this is a gycosylated protein (142-407), hence it has the number of polysaccharide binding motifs. Due to the occurrence of vital domains, *Fein*-LGBP plays major role in crustacean immune system.

Conclusion

In the present study, we identify useful templates that shares significant similarity with *Fein*-LGBP. Interestingly, one of the models derived from comparative modeling in MODELLER9V8 (minimum energy) was validated and displayed several meaningful features like secondary structure, charge distribution, conserved residues engaged in non-bonded interaction. LGBP functional domain prediction also performed it revealed the more knowledge regarding LGBP functional sites. The above work aims to explore the knowledge about the immune related protein *Fein*-LGBP and its

importance in blood clotting mechanism and immune stimulating pathway. The in-silico approach helps researchers to find the appropriate ligands for the specific receptor of the *Fein*-LGBP and it facilitates to develop new ligand that acts like agonist and antagonist for the *Fein*-LGBP protein.

Acknowledgements

This work was supported by Department of Science and Technology (DST), New Delhi, India, under the Project grants code: **SR/FT/LS-075/2008**.

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